RIBONUCLEOTIDE REDUCTASE IN HUMAN BONE MARROW: LACK OF STIMULATION BY 5'-DEOXYADENOSYL B_{12}

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In Lactobacillus leichmannii, the conversion of ribonucleotides to deoxyribonucleotides, catalyzed by ribonucleotide reductase, occurs at the triphosphate level and requires 5'-deoxyadenosyl B_{12} (Abrams, 1965; Blakley and Barker, 1964; Goulian and Beck, 1966). The ribonucleotide reductase in Escherichia coli differs from the enzyme in L. leichmannii in at least two respects: the nucleoside diphosphates are the preferred substrate and no stimulation by a 5'-deoxyadenosyl B_{12} has been demonstrable (Reichard, et al, 1961; Holmgren et al, 1965). It has been speculated for a long time that a vitamin B_{12} derivative may be involved in this reaction in man (Beck, 1962). This postulated mechanism would account for the impairment in deoxyribonucleic acid synthesis observed in pernicious anemia, a prime clinical example of vitamin B_{12} deficiency. The availability of bone marrow from patients with pernicious anemia offered an opportunity to test in cells severely depleted of B_{12} the effect of 5'-deoxyadenosyl B_{12} on the ribonucleotide reductase activity.

Based on indirect evidence (Metz, et. al. 1968) found no role of B_{12} in the reduction of ribonucleotides in man.

MATERIALS AND METHODS

Bone marrow samples were obtained from three patients with pernicious anemia, whose serum B_{12} level was under 0.8 picograms per ml (normal: 3pg/ml). The morphology was grossly megaloblastic as would be expected in B_{12} deficiency. Mature erythrocytes were removed by sedimentation

and osmotic lysis (Bertino et al, 1963). The nucleated cells were washed twice with 0.15 M saline and homogenized in an equal volume of 0.02 M Tris-HCl buffer, pH 7, with 15 strokes of a Potter Elvehjem homogenizer. The homogenate was centrifuged at 20,000 g for 45 minutes. The supernatant solution was subjected to gel filtration on Sephadex G-25. The ribonucleotide reductase assay mixture of 120µl contained 1 µmole potassium phosphate buffer pH 7, 250 mµmoles adenosine triphosphate (Calbiochem), 50 mµmoles magnesium acetate, 5 mµmoles ferric chloride, 750 mµmoles dithiothreitol (Calbiochem), 20 mµmoles cytidine-5'-diphosphate-2-^{14}C; specific activity 21.8 mc per mmole (Schwarz Bioresearch) and approximately 1 mg of protein. This mixture which is essentially that of Moore and Reichard (1964), was incubated at 37°C for 30 minutes. The reaction was terminated by boiling for 2 minutes. After centrifugation, the supernatant solution was treated with bacterial alkaline phosphatase (Worthington) (34 units/ml) to degrade nucleotides to nucleosides. Deoxycytidine and deoxyuridine were separated from cytidine, uridine, cytosine and uracil by two-dimentional descending paper chromatography using appropriate carriers. The first solvent system employed isopropyl ether: n-butanol: 98% formic acid (30:30:20). The second chromatography was carried out with two solvent systems: Isopropanol: ammonia: 0.1 M boric acid (70:10:20) to isolate deoxycytidine and isopropanol: concentrated hydrochloric acid: water (65:16.6:18.4) for the isolation of deoxyuridine. The radioactivity present in the deoxynucleosides was determined with a Liquid Scintillation Spectrometer (Beckman). Since bone marrow contains an active deoxycytidylate deaminase (Maley and Maley, 1959), the specific activity of ribonucleotide reductase was expressed in µµmoles of deoxycytidine and deoxyuridine formed per mg protein per hour. 5'-deoxyadenosyl B_{12} (B_{12} coenzyme) was purchased from Calbiochem; hydroxyurea was obtained from Sigma.

RESULTS AND DISCUSSION

The data shown in Table I indicate that like the enzyme in E. coli

TABLE I

Requirements for Nucleotide Reductase Activity of Human Megaloblastic Bone Marrow

	Deoxycytidine and Deoxyuridine formed ($\mu\mu$moles/mg protein/hour)
Complete	55
Minus Adenosine triphosphate	15
Minus Dithiothreitol	0

TABLE II

Comparative Rates of Reduction of Cytidine-5'-Mono, Di and Triphosphate by Ribonucleotide Reductase

	Deoxycytidine Formed ($\mu\mu$moles/ mg protein)		
Incubation Time (minutes)	10	20	30
Cytidine-5'-monophosphate	10	23	40
Cytidine-5'-diphosphate	26	40	51
Cytidine-5'-triphosphate	13	20	41

The assay mixture was the same as described under METHODS except for the change in ribonucleotides.

bone marrow ribonucleotide reductase has a requirement for adenosine triphosphate and a reduced thiol compound. Unfortunately, because of the limited supply available, purification of the B_{12} deficient marrow enzyme could not be taken beyond the removal of endogenous substrates by gel filtration.

Cytidine diphosphate was used as the substrate since the reaction rate with this compound was consistently more rapid than when cytidine

mono- or triphosphate was used (Table II). This experiment was performed with crude enzyme containing phosphatases; it therefore only offers suggestive evidence for the diphosphate as a preferred substrate. A similar specificity was described in partially purified ribonucleotide reductases from Novikoff hepatoma (Moore and Reichard, 1964) and mouse spleen (Silber and Fujioka, 1969).

Table III shows that the ribonucleotide reductase activity in B_{12} deficient bone marrow was not stimulated by the addition of several levels of 5'-deoxyadenosyl B_{12}. Cyanocobalamin was also added without

TABLE III

The Effect of 5'-Deoxyadenosyl B_{12} on Ribonucleotide Reductase Activity of Human Megaloblastic Bone Marrow

	Deoxycytidine and Deoxyuridine Formed ($\mu\mu$moles/mg protein/hour)					
5'-deoxyadenosyl B_{12} concentration (M)	0	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Sample 1	64	56	70	78	72	
Sample 2	8	2	8	2	3	
Sample 3	78				64	54
L. leichmannii*	29	1679	1903	2063	2051	1464
L. leichmannii plus Sample 1*		1462	1974	1904		
Sample 1*	14	12	10	9		

* These experiments were performed with the following assay mixture: 1 μmole potassium phosphate buffer, pH 7, 2.5 μmoles magnesium acetate, 120 mμmoles deoxyadenosine triphosphate, 5 μmoles reduced lipoate, 10 mμmoles cytidine-5'-triphosphate-2-^{14}C, specific activity 20.6 mC per mmole and approximately 1 mg of protein. Final volume was 130 μl. Mixing experiment contained 0.75 mg of bacterial protein and 0.31 mg of protein from Sample 1 per tube. Specific activity is expressed as $\mu\mu$moles deoxycytidine/mg protein/20 minutes.

effect. The bone marrow enzyme resembles the E. coli system in this respect, as well as in its substrate specificity. As indicated in the Table, L. leichmannii ribonucleotide reductase was stimulated by this B_{12} coenzyme. A mixing experiment of the human and bacterial enzymes showed that the 5'-deoxyadenosyl B_{12} was not inactivated by the bone marrow extract.

Ribonucleotide reductase in megaloblastic bone marrow was inhibited by hydroxyurea, which has been reported as a specific inhibitor for this enzyme in E. coli (Krakoff et al, 1968) and animal systems (Elford, 1968). There was a 33% inhibition with 10^{-4}M and 88% inhibition with 10^{-3}M hydroxyurea. The demonstration that this inhibitor is also active in a human bone marrow system may be useful in designing in vitro studies to evaluate the response to cells from patients who are sensitive or resistant to this chemotherapeutic agent.

SUMMARY

Assays carried out with B_{12} deficient human bone marrow do not support a participation of 5'-deoxyadenosyl B_{12} in the ribonucleotide reductase reaction in man. Hydroxyurea was found to inhibit this enzyme. The human material also resembles the ribonucleotide reductase from E. coli in its substrate requirements.

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